

Cytokine Concentrations in Bronchoalveolar Lavage Fluid from Horses with Neutrophilic Inflammatory Airway Disease

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Background: Multiple cytological patterns occur in bronchoalveolar lavage fluid (BALF) of horses with inflammatory airway disease (IAD). Only few data on BALF cytokine profiles are available for horses with IAD, and are limited to mRNA expression.

Hypothesis/Objective: Cytological profiles of IAD are associated with different BALF immunological pathways. To investigate BALF cytokine concentrations in a large number of horses with neutrophilic IAD.

Animals: One hundred and thirty-eight client-owned Standardbred racehorses in active training.

Methods: Prospective observational study. BALF samples were obtained from left and right lungs. Interleukin (IL)-4, interferon (IFN)- γ , and tumor necrosis factor (TNF)- α concentrations were determined by ELISA.

Results: Fourteen horses had normal BALF cytological profiles and 56 exhibited evidence of bilateral neutrophilic IAD. Twenty-four horses showed BALF with, respectively, IAD- and CTL consistent cytology and were excluded; as were 44 horses because of evidence of pulmonary hemorrhage. TNF- α (56 ± 115 pg/mL; $P = .034$) and IFN- γ concentrations (104 ± 247 pg/mL; $P = .044$) were significantly higher for IAD horses, compared with controls (respectively 19 ± 41 and 80 ± 116 pg/mL). Horses with 'neutrophil' subtype had significantly higher IFN- γ concentrations (110 ± 154 pg/mL), than 'neutrophil/metachromatic' (56 ± 54 pg/mL; $P = .028$) and 'neutrophil/metachromatic/eosinophil' subtypes (44 ± 23 pg/mL; $P = .012$).

Conclusions and Clinical Importance: Cytokine concentrations in BALF suggested that neutrophilic IAD is associated with activation of the innate immune system and a possible T-helper (Th)-1 polarized response. This study also suggested that immunological pathways vary according to cytological IAD subtypes.

Key words: Airway inflammation; ELISA; Th1/Th2; Equine.

Inflammatory airway disease (IAD) is a syndrome defined as a nonseptic lower airway condition affecting horses of any age, which usually exhibit subtle clinical signs at rest.¹ Inclusion criteria for IAD comprise (1) poor performance, exercise intolerance, or coughing, with or without excess tracheal mucus; (2) pulmonary dysfunction or cytological evidence of inflammation on bronchoalveolar lavage fluid (BALF). Exclusion criteria are either increased respiratory effort at rest or evidence of systemic signs of infection.¹ The cytological profile of horses with IAD might reveal an increased total nucleated cell count and a very heterogeneous cell population, with mild neutrophilia or lymphocytosis or, alternatively, increased mast cells or eosinophil counts.^{1,2}

Abbreviations:

BAL	bronchoalveolar lavage
BALF	bronchoalveolar lavage fluid
CTL	control
EIPH	exercise-induced pulmonary hemorrhage
IAD	inflammatory airway disease
IFN	interferon
IL	interleukin
RAO	recurrent airway obstruction
Th	T-helper
TNF	tumor necrosis factor

Multiple BALF cytological patterns in horses with IAD are specifically associated with particular clinical or functional response of the airways. Coughing is associated with significantly higher neutrophil percentages in BALF, compared with noncoughing horses.³ Both mast cell³ and eosinophil⁴ percentages in BALF are correlated with airway hyperresponsiveness, as determined by histamine induced broncho-provocation; these cells also are, respectively, associated with a reduced respiratory compliance and an increased respiratory resistance of the lower airways.⁵ The possibility of IAD subtype dependant biological responses warrants further investigations.

Unlike recurrent airway obstruction (RAO), a condition associated with severe neutrophilic airway inflammation,⁶ very little is known about the molecular pathways and the role of immune system in the pathogenesis of IAD. Characterizing the immunological mechanisms involved in IAD would allow both a more precised diagnosis and the development of appropriate therapeutic options in the future. Several

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Submitted October 22, 2013; Revised May 20, 2014; Accepted August 25, 2014.

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DOI: 10.1111/jvim.12464

studies have investigated cytokine mRNA expression in BALF of horses with IAD,⁷⁻⁹ with variable results in terms of T-helper (Th) lymphocyte polarization. Cytokine mRNA profiles in BALF appear to vary according to the cell types characterizing IAD, particularly neutrophils and metachromatic (mast) cells.^{7,9} All previous studies investigating immunological profiles of IAD were, however, performed on a limited number of horses and based only on mRNA expression,⁷⁻¹⁰ which might be subjected to posttranscriptional regulation and then not truly reflect protein concentrations.

The aim of this study was to investigate the pattern of cytokine concentrations in BALF samples of a large number of horses with IAD. Both interleukin (IL)-4 and interferon (IFN)- γ were investigated, as being archetypical cytokines for, respectively, Th2- and Th1-type responses, and tumor necrosis factor (TNF)- α as pro-inflammatory cytokine reflecting innate immune response. The trial was designed to (1) compare BALF cytokine concentrations in controls and horses with cytological evidence of neutrophilic IAD; (2) compare BALF cytokine concentrations among samples with various IAD cytological subtypes; and (3) determine correlations between cytokine concentrations and BALF cell proportions.

Materials and Methods

Horses

As previously described,¹¹ 138 Standardbred trotters (76 geldings, 58 females and 4 males), aged 4.0 (3.0–6.0) years, were included in this prospective, observational study. Horses were housed in 11 training stables, and were sampled during summer race meetings in 2011 (64 horses) and 2012 (74 horses). None of the horses exhibited clinical signs of respiratory disease at rest (ie, cough or increased respiratory effort) or had exercise intolerance. They were all involved in active training and had raced within less than 1 month before sampling. Before any procedure, each horse was submitted to a thorough clinical examination to insure that no obvious clinical abnormality was present. The study was approved by the regional Animal Ethic Committee (no. CEEA.2012.179) and all owners signed a consent form.

BAL Procedure

BAL was performed at rest using a flexible 3.2 m long, 12.8 mm tip diameter videoendoscope,^a which was randomly introduced within the left or the right lung, according to the previously published procedure.¹¹ A total of 250 mL of sterile isotonic saline solution was instilled via the endoscope biopsy channel, which was prefilled with 20 mL of saline. Two boluses of 125 mL each were instilled, and aspiration was immediately performed manually after each one. The first 20 mL of aspirated liquid, corresponding to the residual volume within the endoscope biopsy channel, were systematically discarded. Following the BAL procedure in one lung side, the endoscope channel was flushed with a 20 mL bolus of isotonic saline. The endoscope was then moved backup to the carina, introduced in the contralateral lung and lavage procedure was repeated. Aliquots of BALF were pooled and kept in EDTA tubes at room temperature for cytological evaluation within 24 hours. All BALF samples for ELISA were stored at -80°C until analysis.

BALF Cytology and Case Definition

All laboratory analyses were conducted in a blinded manner. At reception in the laboratory, 200 μL of fluid were immediately centrifuged ($80 \times g$, 10 minutes)^b and stained with May-Grünwald-Giemsa. Left and right samples were processed simultaneously for each horse. Differential cell count was performed on 300 cells, excluding epithelial cells.

Horses were considered as controls (CTL) when BALF cytological profiles from both lungs revealed $\leq 5\%$ neutrophils amongst leukocytes.^{1,2} Horses for which any BALF exhibited proportions above this cutoff value were considered to have cytological evidence of neutrophilic IAD. In addition, BALF proportions of metachromatic cells and eosinophils (respectively $\geq 2\%$ and $\geq 1\%$ among leukocytes) were also used for characterizing IAD cytological subtypes. Similarly, the ratio of BALF hemosiderophage/macrophage $> 20\%$ was considered as cytological evidence of previous episode(s) of exercise-induced pulmonary hemorrhage (EIPH).¹²

Quantification of BALF Cytokines and BALF Dilution

After concentrating the BALF 10-fold,^c TNF- α , IFN- γ , and IL-4 were measured by ELISA,^d according to the manufacturer's instructions with minor modifications. Briefly, the concentration of detection antibody for TNF- α was set at 300 ng/mL instead of 200 ng/mL; incubation with Streptavidin-HRP and TMB was also increased to 30 minutes instead of 20 minutes, as previously published.¹³ Measures were systematically performed in duplicates. According to previous recommendations,¹⁴ acellular BAL components were expressed as concentration per volume recovered. The range of quantification in BALF was 3.6–500 pg/mL for TNF- α , and 31–2000 pg/mL for IL-4 and IFN- γ .

Urea concentrations were determined in left and right BALF and serum samples;^e and the ratio (BALF/serum) calculated. This served only as an indicator of BALF dilution.¹⁵ and was not employed for normalization (correction) of cytokine concentrations.¹⁴

Statistical Analyses

Arbitrary values corresponding to the lowest and the highest standard concentration were attributed to samples with, respectively, undetectable cytokines and optical density values exceeding the range of quantification. Continuous data distributions were not normally distributed as assessed by Shapiro-Wilk *W*-test;^f and nonparametric tests were then used for further investigations. Differences between groups for each variable were evaluated either by Mann-Whitney or Kruskal-Wallis with Dunn's posthoc tests. Correlations were evaluated by Spearman's correlation coefficient. Data are presented as median (1st–3rd quartile), unless stated otherwise. A difference of $P < .05$ was considered significant for all analyses.

Results

BALF Cytology, Characterization, and Sample Status

Left and right lungs were sampled in 138 horses. Among these, 44 horses met the cytological inclusion criteria for EIPH and were excluded from the present study, as were 24 horses showing BALF with, respectively, IAD- and CTL consistent cytology (Fig 1). A total of 70 horses (140 BALF samples) was then

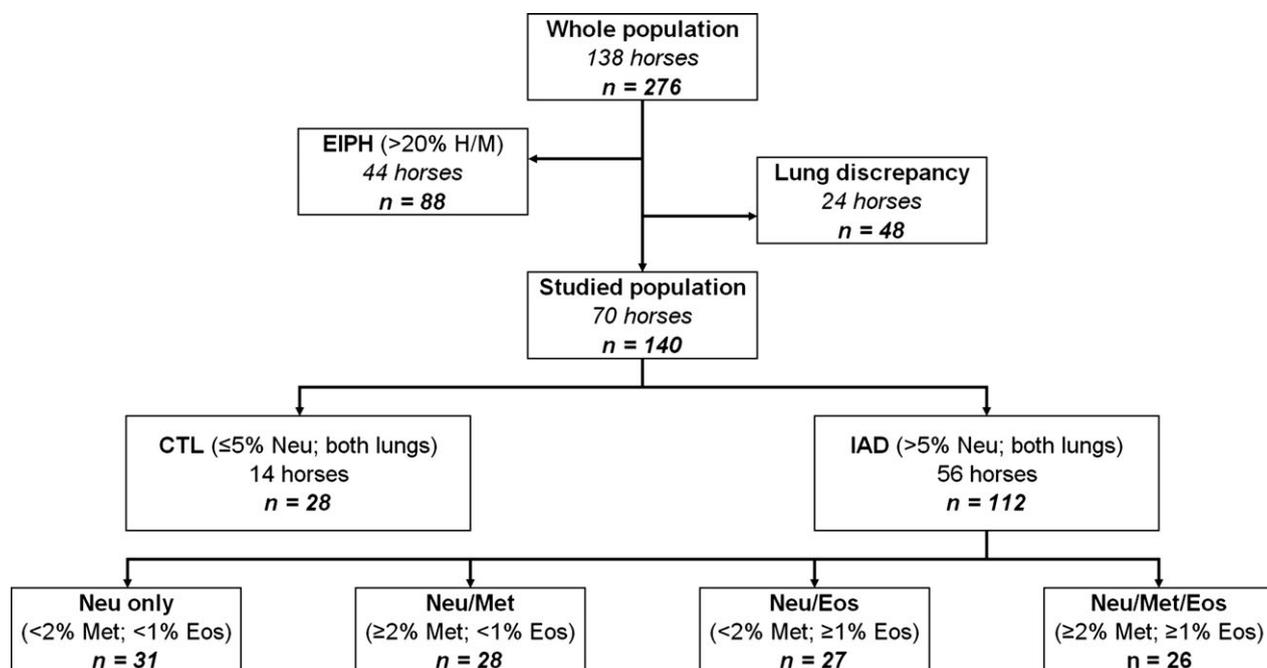


Fig 1. Diagram with breakouts for the 276 bronchoalveolar lavage fluid (BALF) samples according to their cytological profiles. CTL, control; IAD, inflammatory airway disease; EIPH, exercise-induced pulmonary hemorrhage; H/M, haemosiderophages/macrophages; Neu, neutrophils; Met, metachromatic cells; Eos, eosinophils. 'Lung discrepancy' means horses with IAD consistent cytology from one lung and CTL consistent cytology from the contralateral lung.

investigated for cytokine concentrations, among which 14 were CTL and 56 horses had cytological evidence of neutrophilic IAD from both sampled lungs.

Volume of BALF recovered, dilution factor and total cell counts were not significantly different among groups (Table 1). Variable combinations of abnormal BALF cytological profiles were present for the 56 horses of the IAD group (Fig 1). Among these 112 samples with >5% neutrophils, 31 had no other abnormality, 28 also had $\geq 2\%$ metachromatic cells, 27 had $\geq 1\%$ eosinophils and 26 samples had increased proportions of both metachromatic cells and eosinophils.

BALF Cytokine Concentrations

BALF concentrations of TNF- α ($P = .034$) and IFN- γ ($P = .044$) were significantly higher for horses in the IAD group when compared with controls (Fig 2). IAD horses with 'neutrophil' subtype had significantly higher concentrations of IFN- γ , when compared with horses with 'neutrophil/metachromatic' ($P = .028$) and 'neutrophil/metachromatic/eosinophil'

($P = .012$) subtypes (Fig 3). No significant difference was found for IL-4 concentrations in any group comparisons.

Correlations

No significant association was found between total or differential cell counts and any of the investigated cytokines, while IFN- γ and TNF- α concentrations in BALF were significantly correlated ($r = 0.495$, CI 0.353–0.615; $P < .001$).

Discussion

In this study, cytokine concentrations in BALF were investigated in non-RAO horses with lower airway inflammation. Although a large overlap between groups was present, BALF concentrations of both IFN- γ and TNF- α were significantly higher in horses with neutrophilic 'IAD' compared to controls. Concentrations of IFN- γ also differed between IAD subpopulations, the higher concentrations being observed for

Table 1. Characteristics of the 140 bronchoalveolar lavage fluid (BALF) samples, from horses classified as being controls (CTL), or having cytological evidence of inflammatory airway disease (IAD). Data are presented as median (1st–3rd quartile).

Equine Status	Number of Samples	Volume Recovered (mL)	Dilution Factor	Total Cell Count (/mm ³)
CTL	28	117 (97–129)	0.0160 (0.0123–0.0253)	220 (150–300)
IAD	112	109 (100–124)	0.0173 (0.0121–0.0270)	190 (150–255)

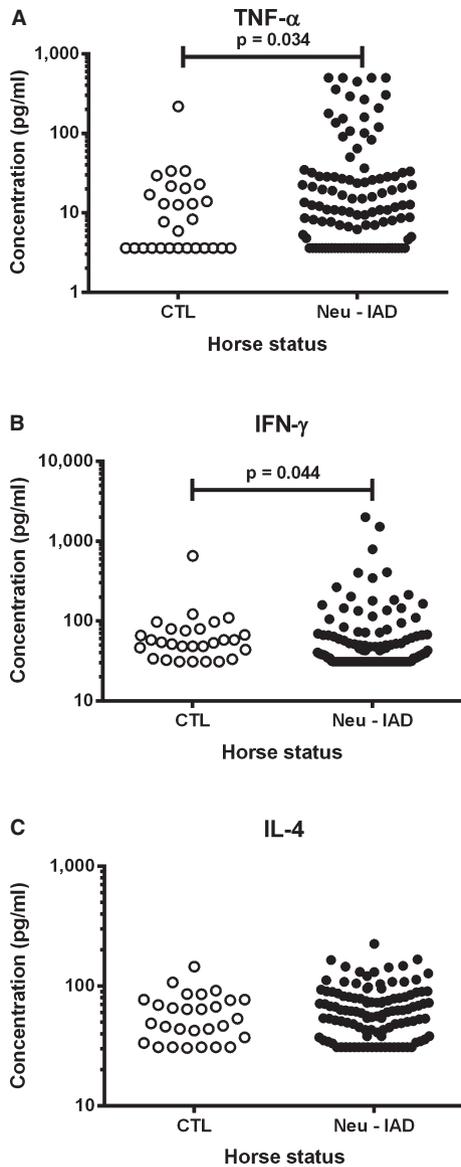


Fig 2. Concentration of (A) tumor necrosis factor (TNF)- α , (B) interferon (IFN)- γ and (C) interleukin (IL)-4 in 140 bronchoalveolar lavage fluid (BALF) samples, from horses classified as being controls (CTL; cytologies with $\leq 5\%$ neutrophils among leukocytes; $n = 28$) or having cytological evidence of inflammatory airway disease (IAD; $>5\%$ neutrophils; $n = 112$).

'neutrophil' subtype compared to other cellular subtypes.

Horses Recruitment and Case Definitions

In this study, BALF samples were collected in left and right lungs of a large population of horses ($n = 138$). This allowed enlarging considerably the number of samples simultaneously analyzed, the final investigations being performed in a total of 70 horses. As a comparison, previous studies on BALF mRNA cytokine expression in IAD were based on overall 27–38 horses,^{7–9} hence limiting the statistical power of the

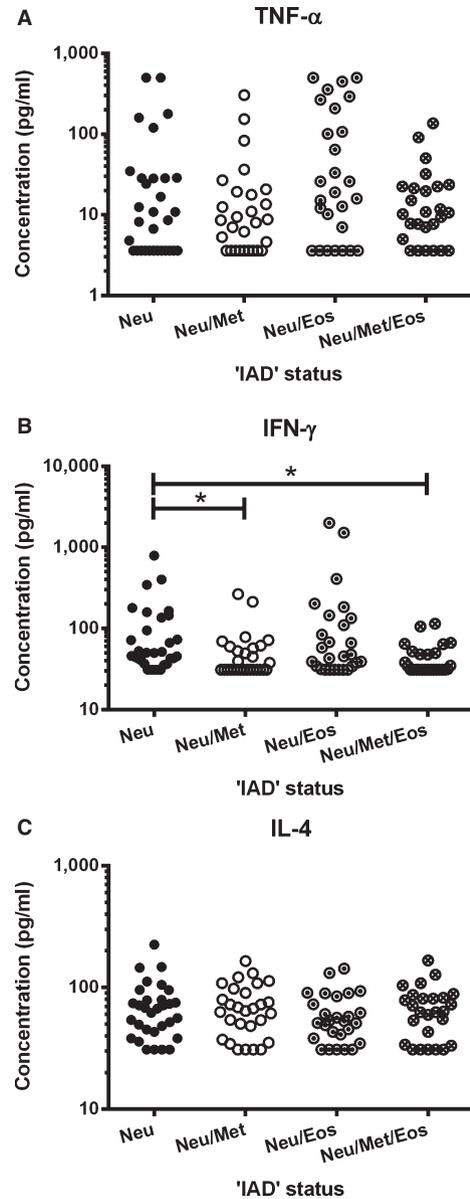


Fig 3. Concentration of (A) tumor necrosis factor (TNF)- α , (B) interferon (IFN)- γ and (C) interleukin (IL)-4 in 112 bronchoalveolar lavage fluid (BALF) samples, from horses with cytological evidence of inflammatory airway disease (IAD), based on increased proportions of neutrophils only (Neu; $>5\%$ amongst leukocytes; $n = 31$), neutrophils and metachromatic cells (Met; $\geq 2\%$; $n = 28$), neutrophils and eosinophils (Eos; $\geq 1\%$; $n = 27$), or neutrophils and metachromatic cells and eosinophils ($n = 26$). Stars indicate significant differences ($P < .05$).

tests, particularly when subpopulations of IAD horses were compared.

The cytological definition of IAD largely varies among studies, making comparisons difficult. We then decided for discriminating CTL and IAD groups, to use the most restrictive (consensual) definition.^{1,2} The phenotype of IAD horses being investigated might also vary among studies, and partially explain some of the controversies among the published results. Horses were

sampled in the field by the authors,⁷ or travelled before investigation, as referred to the veterinary hospital for abnormal respiratory noise, poor performance⁸ or exercise intolerance.⁹ BALF samples from several horses were collected after a strenuous treadmill exercise;⁸ while some other horses, even being sampled at rest, had red tinged BALF indicating recent bleeding.⁹ It was decided in this study to sample horses at rest, since the possibility of cytological modifications of BALF in response to exercise is still controversial,^{16,17} and cytokine mRNA expression might also be influenced by the previous exercise.¹⁸ Similarly, all samples with any evidence of EIPH were excluded from this study, to avoid the uncharacterized immune response potentially induced by the ruptured alveolar capillary walls.

Airway immune response might notably involve T cells, alveolar macrophages, and bronchial epithelial cells (reviewed by Pirie).¹⁹ Systemic inflammatory changes in horses with heaves have, however, been recently described,²⁰ warranting further local (airways) and systemic investigations in IAD affected horses. In this study, cytokine concentrations were indeed measured in BALF samples only. In the same manner, samples from left and right lungs were previously found not to be equivalent, according to linear regression between cell percentages from left and right lung BALF.¹¹ Regarding the final diagnosis of IAD, a fair agreement only is also observed among lungs ($\kappa = 0.30$; CI 0.09–0.50).¹¹ In this study, horses with discrepancy between lungs when considering cytological profiles were then excluded from the statistical analyses, to avoid BALF samples with CTL consistent cytology to be included in the IAD group of horses.

Main Cytokine Profiles

Similar to previous findings on RAO affected horses,^{21–24} contradictory results are observed for IAD with regard to the Th1/Th2 polarized response being involved. Data from this study might suggest a predominant Th1-cell response, while cytokine mRNA expression in previous studies revealed either no Th polarization,⁸ or a Th2-cell predominance,⁷ or a mixed Th1/Th2 response.⁹

TNF- α concentrations in BALF suggested the involvement of pulmonary innate immune response in the pathogenesis of IAD, as previously demonstrated by mRNA expression in horses with either IAD^{8,9} or RAO.²² The statistically significant results from this study should however be interpreted with caution in a clinical setting, since large overlaps between groups were actually present for cytokine concentrations. Although increased concentrations of IFN- γ also were coherent with previous data from both IAD⁹ and RAO,^{21,22} IL-4 concentration in BALF was surprisingly not modified by any condition. Since the horses being sampled might be at various stages of evolution for IAD, a possible hypothesis could be a temporal association with immune response obfuscating the

role of the Th2 pathway. This cytokine is indeed significantly up-regulated in RAO horses^{22,24} as well as IAD horses with increased proportions of metachromatic cells in BALF.⁹ These studies however reported mRNA concentrations only, which might not be reflective of biologically active cytokine concentrations.

IAD Cytological Subtypes

No correlation was found in this study between either cell counts or percentages and BALF cytokine concentrations, which might be because of the multiple cellular sources of cytokines. On the other hand, mRNA expression of both IL-4 and IFN- γ correlates significantly with proportions of metachromatic cells.⁹ In another study, multiple significant correlations were also found between BALF cell types and cytokine expressions;⁷ correlation coefficients were however not provided.

Being present with increased proportions in at least one lung for 120 of the 138 horses with IAD, neutrophils were largely the predominant BALF cell population characterizing this syndrome in this study. No prominent clinical signs of respiratory disease have however been reported, unlike previous findings of an association between airway neutrophilia and chronic cough (>3 weeks).³ Since one of the inclusion criteria of this study was to be in active training and racing at the time of sampling, such chronically coughing horses were however unlikely to be investigated, somehow biasing the investigations toward a subclinical airway inflammation.

Protein concentrations of IFN- γ in BALF were significantly lower when increased proportions of metachromatic cells and neutrophils were simultaneously present, compared to increased proportions of neutrophils only. These results might suggest a down-regulation of Th1 response, possibly induced by Th2 cells. BALF concentrations of IL-4, mainly secreted by mast cells,²⁵ were however not significantly modified; while IL-10, a potent inhibitor of IFN- γ also secreted by mast cells,²⁶ has not been investigated in this study. Conversely, IL-4 mRNA is less expressed in BALF of 'neutrophilic' IAD compared to 'metachromatic' IAD, while IL-10 mRNA expression is not significantly different among these IAD subtypes.⁷

A nonnegligible number of horses with neutrophilic IAD (65/120) also exhibited increased proportions of BALF eosinophils from at least one lung in this study. Being significantly associated with abnormal lung function,^{4,5} these cells were suggested to be responsible for one of the IAD subtypes.^{1,2} While not investigated in the previous studies on IAD,^{7–9} no significant influence of eosinophil proportions on BALF cytokine concentrations was detected in this study. Similarly, neither distinct clinical signs nor significant modifications of IL-4 and IL-5 mRNA expression in BALF occur in horses with transient pulmonary eosinophilia.²⁷

Limitations of the Study

Quantitative polymerase chain reaction (qPCR) for mRNA expression was not performed, since BALF samples have not been appropriately conditioned for RNA preservation. Comparisons between mRNA expression and subsequent protein concentrations might however be relevant in such studies, as posttranscriptional regulation might apply. Indeed, inconsistencies have previously been reported in BALF of healthy horses, where IL-4 mRNA expression was detected, while IL-4 protein was not detected at any time point.²⁸

Moreover, a few cytokines only were investigated, mainly because of the limited number of equine specific reagents currently available and methodological limitations. Using multiplex ELISA²⁹ is largely warranted in the near future, especially when a large cohort of samples has to be investigated. As in previous similar studies,^{7–9} whole BALF with mixed cell populations have been investigated; also leading to undetectable cytokine concentrations in many of the samples. Cell isolation should be performed in the future, and cytokines productions analyzed separately for a better characterization of the Th driven response in IAD.

Conclusion

The results being obtained suggested neutrophilic IAD to be associated with a stimulation of the innate immune system and a possible Th1 polarized response. This study also suggested, at the protein level, that BALF immunological profiles might vary according to the IAD cytological subtypes. Further studies are required to determine the specific cytokine signatures associated with the different IAD phenotypes of athletic horses.

Footnotes

^a VO 320; Optomed, Les Ulis, France

^b Shandon Cytospin; Thermo Scientific, Waltham, MA

^c Amicon Ultra-4 3K; Merck Millipore, Billerica, MA

^d DuoSet; R&D Systems, Minneapolis, MN

^e QuantiChrom Urea Assay; BioAssay Systems, Hayward, CA

^f Prism 6.04; GraphPad, La Jolla, CA

Acknowledgments

The authors thank Marie-Pierre Toquet, Vincent Gennevieve, Ludovic Petit and Claudine Hary for their kind collaboration. This work was supported by 'Hippologia' Foundation.

Conflict of Interest Declaration: The authors disclose no conflict of interest.

Off-label Antimicrobial Declaration: The authors declare no off-label use of antimicrobials.

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